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Review

Strategies for treating disorders of the mitochondrial genome

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Abstract

Defects of the mitochondrial genome are a significant cause of disease. Patients suffer from a wide variety of clinical presentations, ranging from fatal infantile disease to mild muscle weakness. Most disorders, however, are characterized by inexorable progression. As mutations often cause defects in several components of the complexes that couple oxidative phosphorylation, this terminal state of oxidative metabolism cannot be readily bypassed by dietary means, leading to the search for novel therapies. In this article, we present the theory behind several concepts and report progress. We also discuss some of the recent difficulties encountered in the progress towards an antigenomc approach to treating mtDNA disorders.

Keywords: Antigenomic hypothesis; mtDNA disease; Treatment of mtDNA disease; Alloptic expression; Mithocondrial tRNA import

1. Introduction

Disorders of the mithocondrial genome have been catalogued and described in detail in several other accompanying reviews. As many of these disorders affect several complexes of the respiratory chain which function together in the terminal stage of oxidative metabolism, standard strategies for bypassing these metabolic lesions do not appear to be possible for the vast majority of patients. Indeed, while many anecdotal reports describe positive prognoses for the occasional patient treated with various vitamins, cofactors or reagents (for reviews see Refs. [1,2]), there remains no reliable treatment or cure for these often devastating disorders. For this reason, many scientists working in the area of mitochondrial DNA disease have begun to consider alternative strategies towards the long-term goal of halting the progression of disease. In this article, we will report the exciting strategies that are being explored and the progress that has been made. We will also describe some of our recent data on an antigenomic approach to treating these disorders and the inherent frustrations that such a long-term strategy can afford.

2. Redesigning mitochondrial genes for expression from the nucleus

Pathogenic point mutations of mtDNA are most commonly found in mt-tRNA or mt-mRNA genes [3]. A possible therapeutic approach is therefore to try and exploit the natural mitochondrial protein and nucleic acid import pathways. The basic concept is simple-identify the mitochondrial gene that is mutated, introduce a wild-type copy of this gene into the nucleus and import normal copies of the gene product into mitochondria from the cytosol. Expression of a gene in a different cellular compartment to its target location in this manner has been termed allotopic expression.

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2.1. Allotopic expression of mitochondrially encoded proteins

Many nuclear encoded gene products targeted and imported into mitochondria possess a short N-terminal amphipathic protein presequence that may be cleaved after import. The polypeptides encoded by mtDNA in the mitochondrial matrix, in contrast, do not require a targeting signal. It has been well described that a number of non-mitochondrial polypeptides can be relocated to the mitochondrial matrix simply by conjugating a targeting sequence to their N-terminus. However, important questions remain. Can mitochondrially encoded gene products be translocated to the matrix if translated in the cytosol and fused to an N-terminal targeting signal? If so, can the imported protein be assembled successfully into a multi-subunit complex involved in oxidative phosphorylation, even when the allotopically expressed protein has to compete with endogenous mutant polypeptides? In addition to engineering the gene to encode a presequence, there is a further problem—the gene must also be modified to take account of differences in the mitochondrial and standard genetic codes. Codon recognition in human mitochondria differs slightly from the standard genetic code—the triplet AUA specifies methionine in mitochondria (in contrast to isoleucine in the cytosol). Critically, AGA and AGG do not specify arginine but are recognized as translation termination codons. Conversely the stop codon TGA in the standard code specifies tryptophan in the mitochondrial genetic code. In 1988, Nagley and colleagues overcame the difficulties of allotopic expression using yeast as a model system. They recoded the mitochondrial *ATP8* gene from *Saccharomyces cerevisiae* and fused it with part of the gene encoding the N-terminal targeting signal of ATPase 9 of the fungus *Neurospora crassa* [4]. Following engineering and insertion of the gene into the nucleus of *S. cerevisiae*, the allotopically expressed ATPase 8 protein was successfully targeted and imported into mitochondria, Remarkably, this polypeptide was also integrated into a fully assembled ATP synthase restoring oxidative phosphorylation to a strain lacking the normal mitochondrial gene product.

This decisive piece of work demonstrated that allotopic expression was a feasible method of restoring coupled respiratory function, at least in yeast. But could this approach be used to restore oxidative phosphorylation in human cells? After many years of trying to repeat this feat in higher eukaryotes by several research groups, Manfredi et al. [5] reported some success; see also Ref. [6]. In a subset of patients with the mtDNA disease, *N*eurogenic *A*taxia and *R*etinitis *P*igmentosa (NARP), the underlying defect in ATP synthase, is caused by a point mutation in the *MTATP6* gene at np 8993 [7]. Manfredi et al. recoded a wild-type copy of the *MTATP6* gene and added presequences from human COXVIII or ATPase 9 polypeptides. Utilizing cell lines carrying the NARP mutation, the chimeric gene encoding the ATPase 9 presequence was introduced by viral transfection into the nucleus and the wild-type fusion protein was reported to partially rescue the biochemical phenotype. Although this exciting result has great potential, rescue of the defect is not only partial, but also temporary. Furthermore, experimental data reported by Oca-Cossio et al. [8] suggest that the spectrum of mtDNA-encoded peptides that can be successfully expressed and integrated into functional mitochondrial enzyme complexes is limited, and it has been previously reported that some transmembrane domains present in mtDNA encoded polypeptides can be refractive to mitochondrial import [9]. Nevertheless, several groups are still motivated to try and identify a more long-term and definitive rescue by allotopic expression.

Another exciting approach is to transfect human cells with a respiratory gene from a different species and to correct the respiratory defect by mitochondrial import of the gene product. Such an approach has been used to partially rescue the same NARP mutation in human cell lines by expression of the nuclear ATPase 6 gene of *Chlamydomonas reinhardtii* [10]. An interesting twist of this concept has been attempted by Yagi and colleagues, using NADH:quinone oxido-reductase (crudely equivalent to mammalian complex 1) from *S. cerevisiae*. The protein is expressed from a single nuclear gene (*NDI1*) and is relatively rotenone insensitive [11]. This yeast gene was been introduced into a variety of cell lines using the adeno-associated virus [12,13]. Resultant transfected cell lines imported the yeast respiratory complex 1 into the mitochondrial matrix and the enzyme was shown to be fully functional. This work was extended by using human cell lines lacking the essential mtDNA-encoded ND4 gene product. Transfectants expressing the targeted NDi1 gene product demonstrated NADH-dependent and rotenone-insensitive respiration, which was antimycin-sensitive, showing the NDi1 protein to be incorporated into an active respiratory chain [14]. Although impressive in concept, NDi1 unfortunately does not pump protons across the inner membrane, and this was reflected in a lowered P/O ratio for NADH-dependent coupled respiration. Therefore, although feasible that NDi1 would be an effective gene therapy for complex I deficiency if expressed in human cells, oxidative phosphorylation would remain less efficient than normal. It must, however, be noted that efficient expression of NDi1 in cell lines devoid of the mtDNA-encoded ND4 gene product restored growth of these cells in galactose media, which requires coupled oxidative phosphorylation [15].

2.2. Allotopic expression of mt-tRNA genes

In many patients with mtDNA disease, the mutation is present in genes encoding mt-tRNAs. How could allotopic expression be used to treat such patients? It has been known for many years that all plant and protist species

import from the cytosol tRNAs that function in mitochondrial translation, although the number and identity differs between species [16–18]. The situation is different in humans. All mt-tRNAs are encoded by the mitochondrial genome and there is no requirement for tRNA import. Despite this, Tarassov, Martin and colleagues embarked upon a series of experiments to examine the possibility of tRNA import as a gene therapy strategy for mitochondrial DNA disease. First, they examined the only tRNA in *S. cerevisiae* that is transcribed from nuclear DNA and then imported into mitochondria, $tRNA^{Lys}$ (tRK1). They then proceeded to demonstrate that tRK1 and derivatives can be imported into isolated human mitochondria [19]. The import required the $tRNA^{Lys}$ to be aminoacylated and for the import reaction to contain the mitochondrial precursor of the yeast lysyl tRNA synthetase (pre-MSK) and other undetermined factors. The same group has recently found that certain nuclear-expressed tRNAs can be targeted and imported into mitochondria of cultured human cells and, excitingly, that the $tRNA^{Lys}$ defect associated with Myoclonic Epilepsy with Ragged Red Fibres (MERRF) can be partially rescued in transmitochondrial cybrids and patient-derived fibroblasts by importing yeast $tRNA^{Lys}$ derivatives from the cytosol [20]. In addition to import of tRNAs, there is evidence to suggest that the RNA components of MRP RNase and RNase P are translocated into mammalian mitochondria [21,22]. Finally, some 5S rRNA co-localises with mitochondria [23] and may well have a currently undescribed function. By identifying the important *cis*-acting elements required for successful RNA import, it is envisaged that novel vectors will be designed to import tRNAs, antisense RNAs or even ribozymes that may be able to modulate levels or expression of mitochondrial transcripts.

The allotopic expression of both mitochondrial proteins and mt-tRNAs is a potentially exciting method for treating patients with defects of the mitochondrial genome. However, the correctly engineered genes must be delivered, recombined into the nucleus and faithfully expressed in a large number of human cells for this approach to be viable.

3. Manipulation of mtDNA heteroplasmy

Many patients suffering from mtDNA defects often harbour subpopulations of mutated and wild-type mtDNA molecules within the same cell and tissue—a phenomenon known as mtDNA heteroplasmy. A reverse of the disease phenotype would result if wild-type copies were propagated at the expense of the mutated mtDNA. But how can the mutated molecules be eliminated and the wild-type copies selectively amplified? A number of approaches are being investigated. A concept known as cell replacement therapy has been considered. This exploits the surprising observation that for some patients, in particular those with sparing myopathies, the pathogenic mtDNA appears to be absent in terminally fated myogenic precursor cells named satellite cells [24,25]. Myotoxins or anaesthetics such as $BaCl_2$, buvipicaine or notexin have been used to destroy fully formed myofibres, leading to activation and repopulation of the muscle bulk by the satellite cells [26,27]. Although this has been successfully performed and wild-type mtDNA levels have been substantially increased in vivo, it is not at all clear as to the amount of tissue that can be regenerated. A second approach is to subject individuals to various exercise regimes, increasing muscle bulk and potentially increasing the amounts of wild-type mtDNA. This approach and the related experimental observations are detailed in one of the following reviews by Taivassalo et al.

Heteroplasmy could also be manipulated directly by the selective degradation of mutated mtDNA or by selective inhibition of replication. Two groups have had some success with the former approach by recognizing that some pathogenic mutations can introduce novel and unique sites for restriction endonucleases. One such example is the A8993G NARP mutation (that can also cause Leigh's disease), which introduces a unique site for the endonuclease *Sma*I. By constructing a gene where the encoded *Sma*I endonuclease possesses an N-terminal targeting presequence, transfectants expressing this chimaera were able to import the new protein into the mitochondrial matrix of cells heteroplasmic for the 8993G mutation, leading to a dramatic loss of the mutated mtDNA [28]. Following a similar method with endonucleases targeted to the mitochondria of human and rodent cell lines [29], Bayona et al. [30] have now begun to assess whether such a targeting strategy can also alter heteroplasmy levels in the tissue of heteroplasmic mice. This method certainly has great potential, but is unfortunately only suitable for mutations that generate new and unique restriction endonuclease sites. In addition, it is limited in a similar manner to several other approaches in that it requires transfection of all cells that are defective in oxidative phosphorylation.

Another potential, and in principle straightforward, mechanism would be to employ factors that influence segregation bias, i.e., induce or overexpress a nuclear gene whose product promotes propagation of wild-type over mutant mtDNA. Although the existence of such factors is implied by the outcome of segregation studies in cultured human cells [31,32] and in the tissue of transgenic mice [33,34], as yet, no such factor has been identified.

Finally, it has been reported that mammalian mitochondria possess a mismatch repair activity that shows no apparent strand bias [35,36]. It is possible that this repair activity could be utilized to repair mutated mtDNA. Although conceptually interesting, this would require import and hybridization of a single stranded 'wild-type' oligonucleotide to the region of mtDNA around the mutation site. This approach would therefore require

transfection of mitochondria in vivo with exogenous nucleic acid, something that has yet to be achieved for the mammalian organelle.

3.1. Antigenomic therapy for disorders of the mitochondrial genome

A very promising method for manipulating levels of heteroplasmy is to identify a molecule that can selectively inhibit replication of mutated mtDNA. Mitochondrial DNA is believed to be replicated and degraded in vivo, even in postmitotic tissues, although it has traditionally been difficult to dissect DNA repair from true replication when data from numerous experiments have been analysed. From studies with cultured cells it appears there is a mechanism for maintaining mtDNA copy number [37]. If an agent could be identified that could selectively inhibit replication of the pathogenic mtDNA, the wild-type copy would propagate over time, rescuing the cellular defect in oxidative phosphorylation and, in theory, halting the disease progression [38]. Quite simply, this is the antigenomic strategy for mtDNA disease. There are three properties that are essential for any antigenomic agent (Fig. 1). First, it must be able to discriminate between pathogenic mtDNA and wild-type mtDNA that in some cases may only differ by one single base pair. Second, the agent must be able to pass across the plasma membrane, be targeted to mitochondria and be imported across two membranes into the matrix, where it must bind to the mutated mtDNA. Third, the molecule must bind irreversibly, as progression of the natural replication or transcription machinery along the DNA template is likely to melt the hybridizing antigenomic agent from the DNA target.

Advances in targeting molecules to mixed-sequence duplex DNA are beginning to be made, as illustrated by recent work with pseudocomplementary peptide nucleic acid molecules [39]. However, as mtDNA replicative intermediates must be single-stranded for a period during replication, it may not be necessary to target duplex mtDNA. Selective hybridization of antigenomic agents to single-stranded DNA could be effective in inhibiting mtDNA replication, particularly if this binding was irreversible. The molecules we have focused on to perform this function are peptide nucleic acids (PNAs), DNA mimics that bind with higher affinity to complementary single-stranded DNA than the comparable DNA oligomer [40]. Several years ago, we showed that PNAs can selectively inhibit the replication of mutated mtDNA (A8344G MERRF mutation) templates in an in vitro run of replication assay [41]. PNA-mediated inhibition of replication has also recently been shown by targeting pUC19 in *E. coli* [42]. Further to the in vitro experiments with the mtDNA templates, targeting of the PNA molecules to mitochondria was then investigated by constructing a PNA conjugated to a mitochondrial protein presequence [43] and also to a triphenylphosphonium cation (TPP). This caged lipophilic cation is able to pass through lipid bilayers and, due to its positive charge, is actively accumulated by mitochondria [44]. Both methods showed co-localisation of the PNA to mitochondria in intact cells, with the latter approach yielding data which was consistent with the PNA gaining access to the mitochondrial matrix. Disappointingly, however, no alteration in the levels of heteroplasmy was observed with cells heteroplasmic for the A8344G MERRF mutation. It was believed from the data that the PNA was present in the mitochondrial matrix but the lack of effect was due to the PNA not being able to access the mtDNA or to the removal of the bound molecule by the replication or transcription machinery. To dissect these possibilities, PNA molecules capable of covalent interactions were designed and synthesized [45]. This new generation of PNA molecules was trifunctional, possessing a photo-activatable cross-linking moiety (benzophenone) allowing covalent attachment to DNA and a triphenylphosphonium group to facilitate targeting to the mitochondria. These molecules did indeed exhibit specific cross-linking to mutated mtDNA templates, but not to wild-type templates under physiological conditions [45].

To assess import competency, these molecules were then subjected to import assays with isolated rat liver mitochondria [46]. Two methods were employed. The first attempted to exploit the triphenylphosphonium moiety by employing a TPP-selective electrode as had previously been used to monitor the import of a TPP-PNA molecule [44]. The optimized candidate PNA molecule from the previous cross-linking work (PNA 6b, Fig. 2a) was analysed along with a number of other TPP-PNA derivatives. Surprisingly none of our TPP-PNA molecules induced a response with the electrode sensitive to TPP, in contrast to previous reports for the PNA conjugated with the TPP alone. Control experiments with the small lipid-soluble TPMP cation confirmed the ion-selective electrode to be functioning correctly (data not shown). TPMP import was dependent on

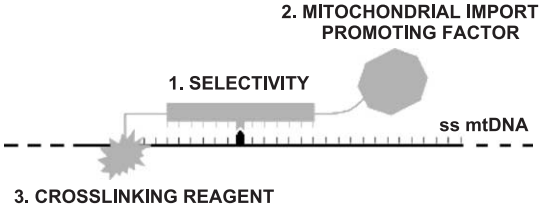

Fig. 1. The optimal antigenomic agent. The figure is a schematic representation of an agent that would be designed to inhibit replication of a mutated mtDNA molecule. 1. Selectivity—The agent must show selective binding to the mutated molecule under physiological conditions. The mutated mtDNA might differ from the wild-type counterpart by only a single nucleotide. 2. Mitochondrial import promoting factor—The agent must also be able to penetrate across the plasma membrane, localise to the mitochondrion and be imported across the outer and inner mitochondrial membrane, accessing the matrix and mtDNA. 3. Cross-linking reagent—Finally, the ideal antigenomic agent would be activatable once bound to the mutated DNA such that it became irreversibly bound. Molecules that have been designed to address each of these requirements are discussed in the text.

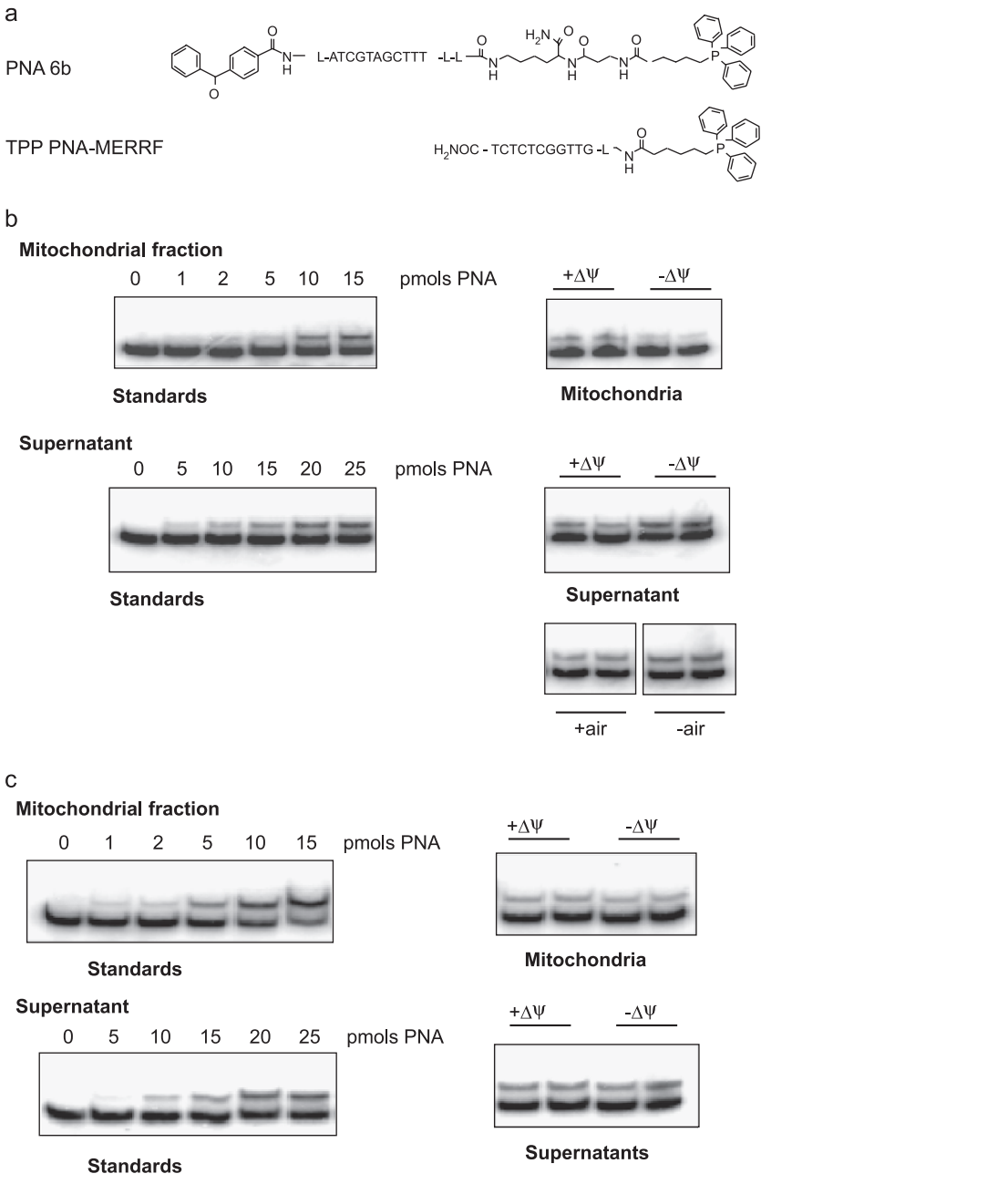

Fig. 2. Consistent import of PNA derivatives into mitochondria is not driven by triphenylphosphonium. (a) Schematic of PNA derivatives, PNA 6b (trifunctional reagent) and TPP-PNA MERRF. For PNA 6b, the PNA sequence is targeted against the rat ND5 gene and has a benzophenone moiety tethered to its N terminus, which can covalently cross-link to DNA when irradiated with UV at 350 nm. The L denotes a linker molecule, (8-amino-3-6-dioxaoctanoic acid unit). The TPP group is conjugated to the C terminus. Other ND5-targeted PNA molecules tested for mitochondrial import were of a similar nature although some possessed the TPP group, alone. The TPP-PNA MERRF molecule has a different PNA sequence to target the mtDNA sequence spanning the A8344G mutation commonly associated with MERRF. It has only the TPP group conjugated to its N-terminus. (b) Mitochondrial import of PNA 6b is negligible. Rat liver mitochondria (100 μg) were incubated in 3-ml import buffer (120 mM KCl; 10 mM HEPES KOH pH 7.2; 1 mM EGTA; 50 μM rotenone; 10 mM succinate) with 1 μM PNA 6b at 25 °C for 5 min in the presence (+ΔΨ) and absence (−ΔΨ) of FCCP, to dissipate the membrane potential. As a second indicator of membrane potential dependent import, uptake experiments were also performed while respiring (+air) and nonrespiring (−air). Mitochondria were centrifuged through oil to separate mitochondrial pellet (imported) from supernatant (unimported). To detect PNA, equal aliquots of the lysed mitochondrial and supernatant fraction (in duplicate) were then hybridized to radiolabelled complementary oligonucleotides and separated through a 16% non-denaturing polyacrylamide gel. The efficiency of uptake could be calculated against standards of known amounts of PNA added to mitochondrial lysate and run in parallel with the import samples. In no import experiment was this necessary, as comparably low levels of PNA derivative were always found associated with the pellet, irrespective of membrane potential. (c) Mitochondrial import of TPP-PNA MERRF is also negligible. Import experiment was performed as detailed in B, except PNA 6b was substituted with TPP-PNA MERRF.

a membrane potential supplied by a respiratory substrate and egress of TPMP occurred on addition of FCCP, an uncoupler of the membrane potential. Surprisingly, 11 separate PNA molecules conjugated with TPP alone or in addition to the benzophenone moiety were all unable to be registered by the TPP electrode.

Original import experiments had relied on visualizing the TPP-PNA derivatives in the mitochondrial matrix or supernatant by using antisera to the triphenylphosphonium moiety [44]. Although the antiserum was very specific, the detection method required substantial amounts of the TPP (~100–200 pmol) to be immobilized. By optimizing a novel gel mobility retardation assay, we were able to increase the sensitivity of TPP detection by two orders of magnitude [46]. The assay employed a radiolabeled oligonucleotide complementary to the PNA sequence. The oligonucleotide when bound to PNA was retarded in its migration through a non-denaturing polyacrylamide gel. By electrophoresing known amounts of PNA derivative to form a standard curve, the assay was used to semiquantitatively detect PNA derivatives imported or associated with isolated mitochondria. Energized rat liver mitochondria were incubated with PNA 6b in the presence or absence of the uncoupler FCCP (Fig. 2b). The mitochondria were then separated from the reaction mixture, lysed and the lysate analysed for the presence of trifunctional PNA. Aliquots of the supernatant following import were also subjected to a similar assay. A small quantity of PNA 6b was associated with the mitochondria during the import assay; however, this amount did not differ on dissipation of the membrane potential. Indeed, for all 11 PNA conjugates subjected to the import assay there was no consistent membrane potential-dependent import into the mitochondria.

There are several possible reasons why there is no membrane potential-dependent association of the TPP-PNAs with isolated mitochondria. Clearly, TPMP or various small uncharged molecules conjugated to TPP can be efficiently imported across both mitochondrial membranes or at least into the inner mitochondrial membrane [47,48]. The extra mass of the PNA molecule, however, undoubtedly has an effect on the diffusion potential of the whole molecule. Surface potential may also have been an inhibitor of association. The presence of fixed negative charges on the surface of the membrane increases the proton concentration in the immediate vicinity of the membrane to be higher than in the bulk phase [49]. The PNA molecules contain cytosine bases, which are likely to become protonated at this lower pH [50]. This would introduce a localised charge in the molecule, possibly promoting weak association and almost definitely preventing it from passing through the membrane. This could potentially be addressed by substituting cytosine monomer for the pseudoisocytosine derivative that cannot be readily protonated [40]. Finally, It may be possible that the electron distribution in the TPP moiety is altered by the presence of the rest of the PNA molecule and, as such, affects the effective ion radius, which would change the activation energy required for transport across the membrane [51].

We must therefore conclude that, at least under the indicated conditions, conjugation of PNAs with the caged lipophilic cation TPP does not promote mitochondrial import of the derivatives. A large range of TPP-PNA derivatives was tested for import competency including some molecules that contained two or three TPP moieties (data not shown), but not one showed any consistent import. Interestingly, a similar lack of mitochondrial import was also noted when cell-penetrating peptides were conjugated with TPP moieties [52]. We are now considering alternative nucleic acid-based chemistries as the template for new antigenomic molecules.

4. DNA import into human mitochondria by a natural mechanism?

A final attractive model should also be considered. Koulintchenko et al. [53] reported the presence of a natural DNA import system into plant mitochondria. The relevant transporter has not been identified, but it is interesting to speculate that such a DNA transport system has been retained in mammalian mitochondria in much the same way as for the tRNA import system [20]. This being the case, all that would be needed to promote DNA import might be a system for delivering the DNA to the vicinity of the mitochondrion. DQAsomes, cationic lipid vesicles onto which DNA can be condensed, have already been shown to enter cells and to localise to mitochondria as a function of the membrane potential [54,55]. Excitingly, on reaching mitochondria, these vesicles have been shown to shed at least some of their DNA load, which might pave the way for mitochondrial transfection in vivo in the future [56].

5. Summary

The progressive nature of mtDNA disorders makes it imperative that we consider all possible treatment strategies. Over the last few years, several ingenious methods have been reported. Those methods that require genetic transformation such as the allotopic expression procedures are very elegant, but there are many practical hurdles to be negotiated. Cell replacement therapy is attractive, but the limits of tissue regeneration are unknown. Perhaps still the most promising long-term therapy, at least for those patients with heteroplasmic mtDNA mutations, is to provide an antigenomic agent as a form of drug. Unfortunately, the difficulties in importing such agents into the mitochondrial matrix have retarded this approach, but the advent of a new series of lipid-soluble nucleic acid monomers may herald a revival in the search for effective antigenomic agents.

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References

[1] B. Marriage, M.T. Clandinin, D.M. Glerum, Nutritional cofactor treatment in mitochondrial disorders, J. Am. Diet. Assoc. 103 (2003) 1029–1038.
[2] D.J. Mahoney, G. Parise, M.A. Tarnopolsky, Nutritional and exercise-based therapies in the treatment of mitochondrial disease, Curr. Opin. Clin. Nutr. Metab. Care 5 (2002) 619–629.
[3] MITOMAP: a human mitochondrial genome database, http://www.mitomap.org.
[4] P. Nagley, L.B. Farrell, D.P. Gearing, D. Nero, S. Meltzer, R.J. Devenish, Assembly of functional proton-translocating ATPase complex in yeast mitochondria with cytoplasmically synthesised subunit 8, a polypeptide normally encoded by the organelle, Proc. Natl. Acad. Sci. 85 (1988) 2091–2095.
[5] G. Manfredi, J. Fu, J. Ojaimi, J.E. Sadlock, J.Q. Kwong, J. Guy, E.A. Schon, Rescue of a deficiency in ATP synthesis by transfer of MTATP6, a mitochondrial DNA-encoded gene, to the nucleus, Nat. Genet. 30 (2002) 394–399.
[6] D.M. Turnbull, R.N. Lightowlers, A roundabout route to gene therapy, Nat. Genet. 30 (2002) 345–346.
[7] I.J. Holt, A.E. Harding, R.K.H. Petty, J.A. Morgan-Hughes, A new mitochondrial disease associated with mitochondrial DNA heteroplasmy, Am. J. Hum. Genet. 46 (1990) 428–433.
[8] J. Oca-Cossio, L. Kenyon, H. Hao, C.T. Moraes, Limitations of allotopic expression of mitochondrial genes in mammalian cells, Genetics 165 (2003) 707–720.
[9] M.G. Claros, J. Perea, Y. Shu, F.A. Samatey, J.L. Popot, C. Jacq, Limitations to in vivo import of hydrophobic proteins into yeast mitochondria. The case of a cytoplasmically synthesized apocytochrome *b*, Eur. J. Biochem. 228 (1995) 762–771.
[10] J. Ojaimi, J. Pan, S. Santra, W.J. Snell, E.A. Schon, An algal nucleus-encoded subunit of mitochondrial ATP synthase rescues a defect in the analogous human mitochondrial-encoded subunit, Mol. Biol. Cell 13 (2002) 3836–3844.
[11] S. de Vries, L.A. Grivell, Purification and characterization of a rotenone-insensitive NADH:Q6 oxidoreductase from mitochondria of *Saccharomyces cerevisiae*., Eur. J. Biochem. 176 (1988) 377–384.
[12] B. Seo, J. Wang, T. Flotte, T. Yagi, A. Matsuno-Yagi, Use of the NADH-quinone oxidoreductase (NDI1) gene of *Saccharomyces cerevisiae* as a possible cure for complex I defects in human cells, J. Biol. Chem. 275 (2000) 37774–37778.
[13] B.B. Seo, E. Nakamaru-Ogiso, T.R. Flotte, T. Yagi, A. Matsuno-Yagi, A single-subunit NADH-quinone oxidoreductase renders resistance to mammalian nerve cells against complex I inhibition, Molec. Ther. 6 (2002) 336–341.
[14] Y. Bai, P. Hajek, A. Chomyn, E. Chan, B. Seo, A. Matsuno-Yagi, T. Yagi, G. Attardi, Lack of complex I activity in human cells carrying a mutation in mtDNA encoded ND4 subunit is corrected by the *Saccharomyces cerevisiae* NADH-quinone oxidoreductase (NDI1) gene, J. Biol. Chem. 276 (2001) 38808–38813.
[15] Y. Bai, P. Hu, J.S. Park, J.H. Deng, X. Song, A. Chomyn, T. Yagi, G. Attardi, Genetic and functional analysis of mitochondrial DNA-encoded complex I genes, Ann. N.Y. Acad. Sci. 1011 (2004) 272–283.
[16] R. Kumar, L. Marechal-Drouard, K. Akama, I. Small, Striking differences in mitochondrial tRNA import between different plant species, Mol. Gen. Genet. 252 (1996) 404–411.
[17] A. Schneider, L. Marechal-Drouard, Mitochondrial tRNA import: are there distinct mechanisms? Trends Cell Biol. 10 (2000) 509–513.
[18] K.E. Glover, D.F. Spencer, M.W. Gray, Identification and structural characterization of nucleus-encoded transfer RNAs imported into wheat mitochondria, J. Biol. Chem. 276 (2001) 639–648.
[19] O.A. Kolesnikova, N.S. Entelis, H. Mireau, T.D. Fox, R.P. Martin, I.A. Tarassov, Suppression of mutations in mitochondrial DNA by tRNAs imported from the cytoplasm, Science 289 (2000) 1931–1933.
[20] O.A. Kolesnikova, F. Goltzene, N. Entelis, Z.M. Chrzanowska-Lightowlers, C. Becker, R.N. Lightowlers, R.P. Martin, I. Tarassov, tRNAs imported from the cytosol can rescue a human mitochondrial DNA MERRF mutation, Hum. Mol. Genet. 13 (20) (2004) 2519–2534.
[21] K. Li, C.S. Smagula, W.J. Parsons, J.A. Richardson, M. Gonzalez, H.K. Hagler, R. Sanders Williams, Subcellular partitioning of MRP RNA assessed by ultrastructural and biochemical analysis, J. Cell Biol. 124 (1994) 871–882.
[22] R.S. Puranam, G. Attardi, The RNase P associated wih HeLa cell mitochondria contains an essential RNA component identical in sequence to that of the nuclear RNase P, Mol. Cell. Biol. 21 (2001) 548–561.
[23] P. Magalhaes, A. Andreu, E.A. Schon, Evidence for the presence of 5S rRNA in mammalian mitochondria, Mol. Biol. Cell. 9 (1998) 2375–2382.
[24] S.K. Bidooki, M.A. Johnson, Z. Chrzanowska-Lightowlers, L.A. Bindoff, R.N. Lightowlers, Intracellular mitochondrial triplasmy in a patient with two heteroplasmic base changes, Am. J. Hum. Genet. 60 (1997) 1430–1438.
[25] K. Weber, J.N. Wilson, L. Taylor, E. Brierley, M.A. Johnson, D.M. Turnbull, L.A. Bindoff, A new mtDNA mutation showing accumulation with time and restriction to skeletal muscle, Am. J. Hum. Genet. 60 (1997) 373–380.
[26] K.M. Clark, L.A. Bindoff, R.N. Lightowlers, R.M. Andrew, P.G. Griffiths, M.A. Johnson, E.J. Brierley, D.M. Turnbull, Reversal of a mitochondrial DNA defect in human skeletal muscle, Nat. Genet. 16 (1997) 222–224.
[27] K. Fu, R. Hartlen, T. Johns, A. Genge, G. Karpati, E.A. Shoubridge, A novel heteroplasmic tRNAleu(CUN) mtDNA point mutation in a sporadic patient with mitochondrial encephalomyopathy segregates rapidly in skeletal muscle and suggests an approach to therapy, Hum. Mol. Genet. 5 (1996) 1835–1840.
[28] M. Tanaka, H.J. Borgeld, J. Zhang, S. Muramatsu, J.S. Gong, M. Yoneda, W. Maruyama, M. Naoi, T. Ibi, K. Sahashi, M. Shamoto, N. Fuku, M. Kurata, Y. Yamada, K. Nishizawa, Y. Akao, N. Ohishi, S. Miyabayashi, H. Umemoto, T. Muramatsu, K. Furukawa, A. Kikuchi, I. Nakano, K. Ozawa, K. Yagi, Gene therapy for mitochondrial disease by delivering restriction endonuclease *Sma*I into mitochondria, J. Biomed. Sci. 9 (2002) 534–541.
[29] S. Srivastava, C. Moraes, Manipulating mitochondrial DNA heteroplasmy by a mitochondrially-targeted endonuclease, Hum. Mol. Genet. 10 (2001) 3093–3099.
[30] M. Bayona, B. Blits, B. Battersby, E. Shoubridge, C. Moraes, Shifting mitochondrial DNA heteroplasmy by mitochondrially targeted restriction endonucleases, Biochim. Biophys. Acta 1657 (2004) 89.
[31] T. Bougeron, D. Chretien, A. Rötig, A. Munnich, P. Rustin, Fate and expression of the deleted mitochondrial DNA differ between human heteroplasmic skin fibroblast and Epstein–Barr virus transformed lymphocyte cultures, J. Biol. Chem. 268 (1993) 19369–19376.
[32] D.R. Dunbar, P.A. Moonie, H.T. Jacobs, I.J. Holt, Different cellular backgrounds confer a marked advantage to either mutant or wild-type mitochondrial genomes, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 6562–6566.
[33] J.P. Jenuth, A.C. Peterson, E.A. Shoubridge, Tissue-specific selection for different mtDNA genotypes in heteroplasmic mice, Nat. Genet. 16 (1997) 93–95.
[34] B.J. Battersby, J.C. Loredo-Osti, E.A. Shoubridge, Nuclear genetic control of mitochondrial DNA segregation, Nat. Genet. 33 (2003) 183–186.

[35] P.A. Mason, E.C. Matheson, A.G. Hall, R.N. Lightowlers, Mismatch repair activity in mammalian mitochondria, Nucleic Acids Res. 31 (2003) 1052–1058.
[36] P.A. Mason, R.N. Lightowlers, Why do mammalian mitochondria possess a mismatch repair activity? FEBS Lett. 554 (2003) 6–9.
[37] M.P. King, G. Attardi, Human cells lacking MTDNA: repopulation with exogenous mitochondria by complementation, Science 246 (1989) 500–503.
[38] Z. Chrzanowska-Lightowlers, R. Lightowlers, D. Turnbull, Gene-therapy for mitochondrial-DNA defects—is it possible? Gene Ther. 2 (1995) 311–316.
[39] V.V. Demidov, E. Protozanova, K.I. Izvolsky, C. Price, P.E. Nielsen, M.D. Frank-Kamenetski, Kinetics and mechanism of the DNA double helix invasion by pseudocomplementary peptide nucleic acids, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 5953–5958.
[40] M. Egholm, O. Buchart, L. Christensen, C. Behrens, S.M. Freier, D.A. Driver, R.H. Berg, S.K. Kim, B. Norden, P.E. Nielsen, PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules, Nature 365 (1993) 566–568.
[41] R.W. Taylor, P.F. Chinnery, D.M. Turnbull, R.N. Lightowlers, Selective inhibition of mutant human mitochondrial DNA replication in vitro by peptide nucleic acids, Nat. Genet. 15 (1997) 212–215.
[42] M.R. Liebling, N.T. Jou, W. Fang, J.S. Louie, Blockade of plasmid replication mediated by peptide nucleic acids, Mol. Biotechnol. 25 (2003) 229–240.
[43] P.F. Chinnery, R.W. Taylor, K. Diekert, R. Lill, D.M. Turnbull, R.N. Lightowlers, Peptide nucleic acid delivery to human mitochondria, Gene Ther. 6 (1999) 1919–1928.
[44] A. Muratovska, R.N. Lightowlers, R.W. Taylor, D.M. Turnbull, R.A.J. Smith, J.A. Wilce, S.W. Martin, M.P. Murphy, Targeting of peptide nucleic acid (PNA) oligomers to miitochondria within cells by conjugation to lipophilic cations: implications for mitochondrial DNA replication, expression and disease, Nucleic Acids Res. 29 (2001) 1852–1863.
[45] G.F. Ross, P.M. Smith, A. McGregor, D.M. Turnbull, R.N. Lightowlers, Synthesis of trifunctional PNA-benzophenone derivatives for mitochondrial targeting, selective DNA binding, and photo-cross-linking, Bioconjug. Chem. 14 (2003) 962–966.
[46] P.M. Smith. The development of an antigenomic approach for the treatment of mitochondrial DNA disease. PhD thesis, Newcastle upon Tyne, UK 2003.
[47] C.V. Coulter, G.F. Kelso, T.-K. Lin, R.A.J. Smith, M.P. Murphy, Mitochondrially targeted antioxidants and thiol reagents, Free Radic. Biol. Med. 28 (2000) 1547–1554.
[48] G.F. Kelso, C.M. Porteous, C.V. Coulter, G. Hughes, W.K. Porteous, E.L. Ledgerwood, R.A.J. Smith, M.P. Murphy, Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties, J. Biol. Chem. 276 (2000) 4588–4596.
[49] F.G. van der Goot, J.M. Gonzalez-Manas, J.H. Lakey, F. Pattus, A 'molten-globule' membrane-insertion intermediate of the pore-forming domain of colicin A, Nature 354 (1991) 408–410.
[50] H. Kuhn, V.V. Demidov, P.E. Neilsen, M.D. Frank-Kamenetski, An experimental study of mechanism and specificity of peptide nucleic acid (PNA) binding to duplex DNA, J. Mol. Biol. 286 (1999) 1337–1345.
[51] A. Ono, S. Miyauchi, M. Demura, T. Asakura, N. Kamo, Activation energy for permeation of phosphonium cations through phospholipid bilayer membrane, Biochemistry 33 (1994) 4312–4318.
[52] M.F. Ross, A. Filipovska, R.A.J. Smith, M.J. Gait, M.P. Murphy, Cell-penetrating peptides do not cross mitochondrial membranes even when conjugated to a lipophilic cation: evidence against direct passage through phospholipid bilayers, Biochem. J. (2004) (in press).
[53] M. Koulintchenko, Y. Konstantinov, A. Dietrich, Plant mitochondria actively import DNA via the permeability transition pore complex, EMBO J. 22 (2003) 1245–1254.
[54] V. Weissig, J. Lasch, G. Erdos, H.W. Meyer, T.C. Rowe, J. Hughes, DQAsomes: a novel potential drug and gene delivery system made from Dequalinium, Pharm. Res. 15 (1998) 334–337.
[55] V. Weissig, V. Torchilin, Cationic bolasomes with delocalised charge centres as mitochondria-specific DNA delivery systems, Adv. Drug Deliv. Rev. 49 (2001) 127–149.
[56] G.G. D'Souza, R. Rammohan, S.M. Cheng, V.P. Torchilin, V. Weissig, DQAsome-mediated delivery of plasmid DNA toward mitochondria in living cells, J. Control. Release 92 (2003) 189–197.